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Biochimica et Biophysica Acta 1495 (2000) 121–124

BIOCHIMICA ET BIOPHYSICA ACTA

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Rapid report

Inhibition of phosphatidylinositol 4,5-bisphosphate-stimulated phospholipase D2 activity by Ser/Thr phosphorylation[1]

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Received 17 November 1999; accepted 7 December 1999

Abstract

Treatment of HeLa cells overexpressing PLD2 with the Ser/Thr-specific protein phosphatase inhibitor, okadaic acid, augmented spontaneous phosphorylation of PLD2 with concomitant inhibition of phosphatidylinositol 4,5-bisphosphate (PIP_2)-stimulated PLD2 activity. Dephosphorylation of the immunoprecipitated, spontaneously phosphorylated PLD2 in COS-7 cells by catalytic subunit of protein phosphatase 1γ1 resulted in the stimulation of the PLD2 catalytic activity. These observations suggest that Ser/Thr phosphorylation regulates PLD2 activity.

Keywords: Phospholipase D; Phosphorylation; Okadaic acid

In a wide variety of types of mammalian cells, phospholipase D (PLD) is activated to produce choline and phosphatidic acid from phosphatidylcholine in response to a large number of extracellular signals through G protein-coupled and tyrosine kinase receptors, being involved in various cell functions such as secretion, mitogenesis, vesicular traffic, and cytoskeletal reorganization [1–5]. Two mammalian PLD isozymes, PLD1 and PLD2, have so far been cloned from human [6,7], rat [8,9], and mouse [10,11]. PLD1, which includes two splice variant forms, is activated by α and β isozymes of protein kinase C and small G proteins, ADP-ribosylation factor and members of Rho family GTPases, in the presence of phosphatidylinositol 4,5-bisphosphate (PIP_2) [12]. On the other hand, PLD2 exhibits high basal activity in the presence of PIP_2, and does not further respond to the PLD1 activators [11]. Because PLD2 has high basal activity in vitro, it has been assumed that in vivo its activity is normally suppressed by its inhibitor(s) [11]. Although α and β synucleins have been found to inhibit PIP_2-stimulated PLD2 activity in vitro [11,13], it remains to be clarified whether they function as PLD2 inhibitors in vivo. Recently, it has been reported that PLD2, which was co-expressed with epidermal growth factor (EGF) receptor in human embryonic kidney fibroblasts, is phosphorylated on Tyr-11 and activated upon stimulation of cells by EGF [14]. Substitution of Phe for this residue, however, did not affect the magnitude of the EGF-stimulated PLD2 activation. These observations suggest that the EGF-induced ty-

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[1] This work was supported in part by research grants from the Ministry of Education, Science, Sports and Culture, Japan.

0167-4889/00/$ – see front matter
PII: S0167-4889(99)00165-2

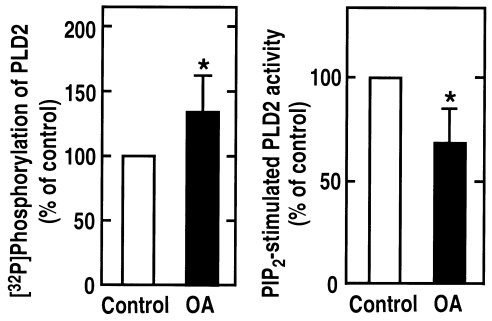

Fig. 1. Stimulation by okadaic acid of phosphorylation of PLD2 in HeLa cells and consequent inhibition of PIP_2-stimulated PLD2 activity. HeLa cells, in which FLAG-PLD2 was transiently expressed, were labeled with [^{32}P]Pi (100 μCi/ml) at 37°C for 3 h. For the last 1 h of labeling, cells were incubated without or with 250 nM of OA. After cells were lysed in a buffer containing 1% Nonidet P-40, solubilized FLAG-PLD2 was immunoprecipitated with the anti-FLAG M2 antibody resin (Sigma). [^{32}P]-phosphorylation of the immunoprecipitated FLAG-PLD2 was analyzed by Fuji BAS-2000 Bioimaging Analyzer after FLAG-PLD2 was separated by SDS-PAGE. The immunoprecipitated PLD2 activity was assayed in the presence of 12 μM of PIP_2 by determining [^{3}H]choline released from the substrate [choline-methyl-^{3}H]dipalmitoylphosphatidylcholine as described previously [15]. Results shown are the mean ± S.D. of five independent experiments with similar results. *, significantly different from the corresponding control as assessed by Student's *t*-test ($P < 0.05$).

rosine phosphorylation of PLD2 is not essential for the regulation of the enzyme activity. In the present study, we have addressed another possibility that PLD2 activity is regulated by its Ser/Thr phosphorylation.

To overexpress FLAG epitope-tagged mouse PLD2 (FLAG-PLD2) in mammalian cells, cDNA of PLD2 was amplified from total RNA prepared from mouse brain by RT-PCR using a set of primers containing *Eco*RI site, and inserted into pTB701-FLAG vector. HeLa cells or COS-7 cells were transfected with 50 μg of this plasmid by electroporation, and cultured at 37°C for 40 h in the presence of 5% fetal calf serum (FCS).

Fig. 1 shows effects of okadaic acid (OA, Calbiochem), the inhibitor of Ser/Thr-specific protein phosphatase 1 and 2A, on phosphorylation and PIP_2-stimulated activity of the transiently expressed FLAG-PLD2 in HeLa cells. When HeLa cells were metabolically labeled with [^{32}P]Pi in the presence of 5% FCS, overexpressed PLD2 was found to be spontaneously [^{32}P]-phosphorylated. OA treatment of cells during metabolic labeling evidently increased the level of phosphorylation, indicating that PLD2 is a substrate for Ser/Thr protein kinase(s). Interestingly, the immunoprecipitated PLD2 activity, which was determined in the presence of PIP_2, was significantly decreased by OA treatment. These results, taken together, suggest that Ser/Thr phosphorylation of PLD2 suppresses the PIP_2-stimulated PLD2 activity.

Since spontaneous phosphorylation of overexpressed PLD2 in COS-7 cells was much greater than that in HeLa cells (data not shown), FLAG-PLD2 was overexpressed in COS-7 cells in the following experiments. When COS-7 cells overexpressing FLAG-PLD2 were treated for the indicated times with 100 μM of pervanadate, the phosphotyrosine-specific phosphatase inhibitor, mobility of PLD2 on SDS-PAGE shifted in a time-dependent manner (Fig. 2A, top panel). This shift was due to the Tyr phos-

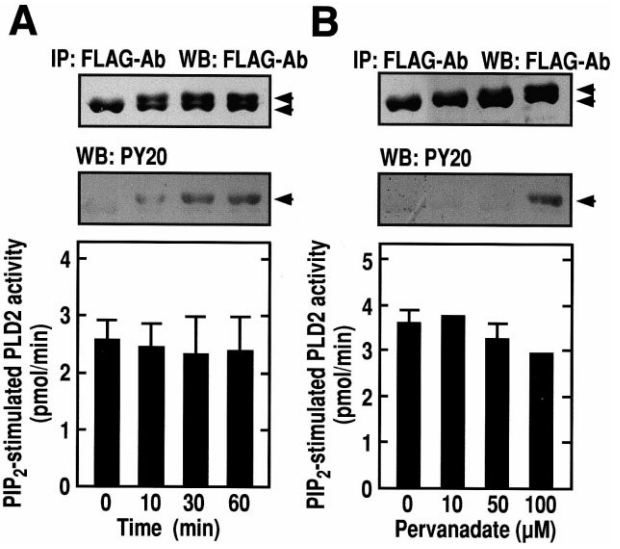

Fig. 2. Tyrosine phosphorylation of PLD2 overexpressed in COS-7 cells does not affect PIP_2-stimulated PLD2 activity. COS-7 cells, in which FLAG-PLD2 was overexpressed, were treated at 37°C with 100 μM of pervanadate for the indicated times (A) or with the indicated concentrations of pervanadate for 1 h (B), then FLAG-PLD2 was immunoprecipitated with the anti-FLAG M2 antibody resin as described in Fig. 1. The immunoprecipitated PLD2 (top panels) and its tyrosine phosphorylation (middle panels) were analyzed by Western blotting with the anti-FLAG M2 antibody (FLAG-Ab) and PY-20, respectively. PIP_2-stimulated PLD2 activity in the immunoprecipitant (bottom panels) was also determined as described in Fig. 1. The *error* represents the differences of duplicate determinations. The results shown are from a single experiment representative of three with similar results.

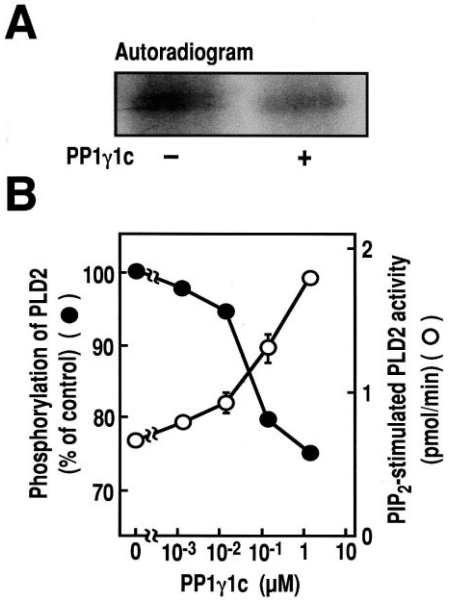

Fig. 3. Inhibition by Ser/Thr phosphorylation of PIP_2-stimulated PLD2 activity. COS-7 cells overexpressing FLAG-PLD2 were labeled with [^{32}P]Pi (30 μCi/ml) at 37°C for 24 h in the presence of 5% FCS, then FLAG-PLD2 was immunoprecipitated with the anti-FLAG antibody resin. The affinity-purified FLAG-PLD2 was then incubated at 25°C for 1 h without or with 1.4 μM (A) or the indicated concentrations of PP1γ1c (B). The level of phosphorylation of PLD2 was analyzed by autoradiography (A) or Fuji BAS-2000 Bioimaging Analyzer (B, closed circles) after SDS-PAGE of the immunoprecipitated PLD2. The immunoprecipitated PLD2 activity in the presence of PIP_2 (B, open circles) was determined as described in Fig. 1. The *error* represents the differences of duplicate determinations. The results shown are from a single experiment representative of three with similar results.

phorylation of PLD2, as detected by the anti-phosphotyrosine antibody PY-20 (Fig. 2A, middle panel). The Tyr phosphorylation of PLD2 was also stimulated in a pervanadate concentration-dependent fashion (Fig. 2B, top and middle panels). However, pervanadate was without effect on the PLD2 activity determined in the presence of PIP_2 (Fig. 2A and B, bottom panels), demonstrating that Tyr phosphorylation does not modulate the PIP_2-stimulated PLD2 activity. These results are consistent with the observation by Slaaby et al. that PLD2 is a substrate for tyrosine protein kinase(s), but the Tyr phosphorylation is not essential for PLD2 activity [14].

To confirm the inhibition of PIP_2-stimulated PLD2 activity by Ser/Thr phosphorylation, PLD2, which was spontaneously [^{32}P]-phosphorylated in and affinity purified with the anti-FLAG antibody resin from COS-7 cells, was treated with the catalytic subunit of human protein phosphatase 1γ1 (PP1γ1c) (Calbiochem), and the levels of phosphorylation and of PIP_2-stimulated PLD2 activity were compared (Fig. 3). When spontaneously [^{32}P]-phosphorylated PLD2 in COS-7 cells was treated with 1.4 μM of PP1γ1c at 25°C for 1 h, phosphorylation level was significantly decreased (Fig. 3A). With increasing concentrations of PP1γ1c, both dephosphorylation of PLD2 and PIP_2-stimulated PLD2 activity were increased with good correlation (Fig. 3B). Thus, Ser/Thr phosphorylation of PLD2 suppressed the PIP_2-dependent PLD2 activity.

The mechanism by which Ser/Thr phosphorylation suppressed the PIP_2-stimulated PLD2 activity can be explained by either inhibition of catalytic activity of PLD2 or the decrease in the affinity of PLD2 for PIP_2. To analyze which mechanism is more likely, concentration dependence of PIP_2 in the activation of the phosphorylated and dephosphorylated PLD2 was examined (Fig. 4). Both phosphorylated and dephosphorylated PLD2s were activated in a PIP_2 concentration-dependent manner with almost the same EC_{50} value (about 2.5 μM PIP_2), leaching a plateau at about 12 μM of PIP_2. However, the maximal stimulation by PIP_2 of the dephosphorylated PLD2 was

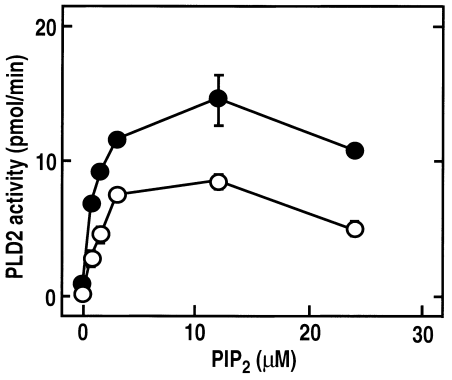

Fig. 4. Mode of inhibition of PIP_2-stimulated PLD2 activation by Ser/Thr phosphorylation. Spontaneously [^{32}P]-phosphorylated PLD2 in COS-7 cells was immunoprecipitated and treated without (open circles) or with 1.4 μM PP1γ1c (closed circles), as described in Fig. 3. The activities of phosporylated and dephosphorylated PLD2 were determined in the presence of the indicated concentrations of PIP_2. The *error* represents the differences of duplicate determinations. The results shown are from a single experiment representative of three with similar results.

higher than that of the phosphorylated PLD2. These results demonstrate that Ser/Thr phosphorylation inhibits the catalytic activity of PLD2 without interference with the affinity for PIP_2.

In conclusion, PLD2 can be phosphorylated on its Ser/Thr residue(s), and the Ser/Thr phosphorylation results in the inhibition of the catalytic activity of PLD2. When HeLa cells co-overexpressing PLD2 and EGF receptors were treated with OA, EGF-stimulated PLD activity was evidently inhibited (data not shown). These observations are consistent with the idea that agonist-stimulated PLD2 activation is regulated, at least in part, by Ser/Thr phosphorylation.

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